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Ultrastructural networks in growth cones and neurites of cultured central nervous system neurons

(cytoskeleton/synaptic vesicles/filopodia/high-voltage electron microscopy/synapse)

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ABSTRACT **We have examined growth cones and neurites of cultured central nervous system neurons by high-voltage electron microscopy. Embryonic chicken retina cells were cultured on polylysine-treated and Formvar-coated gold grids for 2–6 days, fixed, and critical point dried. Growth cones and neurites were examined as unembedded whole mounts. Three-dimensional images from stereo-pair electron micrographs of these regions showed a high degree of ultrastructural articulation, with distinct, nontapering filaments (5–9 nm in diameter) joining both cytoskeletal and membranous components. In the central regions of growth cones, interconnected structures included microtubules, large membranous sacs (up to 400 nm), and irregular vesicles (25–75 nm). A denser filamentous network was prevalent at the edges of growth cones. This network, which frequently adjoined the surface membrane, linked vesicles of uniform size (35–40 nm). Such vesicles often were seen densely packed in growth cone protrusions that were about the size of small synaptic boutons. Prevalent structural interconnections within growth cones conceivably could play a logistic role in specific membrane assembly, intracellular transport, endocytosis, and secretion. Because such processes are not unique to growth cones, the extensive linkages we have observed may have implications for cytoplasmic structure in general.**

Cellular growth and local differentiation are of particular importance in the developing nervous system. Numerous branching processes grow out from immature neurons, often forming thousands of synaptic contacts with other neurons. Motility of these developing dendrites and axons, collectively known as neurites, is restricted to specialized regions at their tips called growth cones. In nerve cell culture, growth cones are readily distinguished by their large, flattened appearance and their many motile filopodia (1, 2). Cellular components are transported to these growing tips, and new membrane is added to neurites specifically at these regions (3–5). Growth cones also are active in micropinocytosis and initiation of retrograde transport (6, 7), processes that appear to be important in the mechanisms of action of developmental signals such as nerve growth factor (8).

The structural basis for growth cone function is poorly understood. To obtain a more detailed picture of growth cones we have examined cultured central nervous system (CNS) neurons by using high-voltage electron microscopy (HVEM). Growth cones, as well as flattened regions along neurites, are particularly amenable to study because they are thin and can be viewed as whole-mount specimens. HVEM analysis of unembedded whole mounts gives a three-dimensional perspective of all major structures within a subcellular space, providing great depth of field and resolution (9). Our studies indicate a surprisingly high level of organization within growth cones and developing neurites, showing abundant linkages between membranous and cytoskeletal components throughout each specimen. The prevalent structural interconnections seen here may have bearing on how adult as well as embryonic cells carry out specific intracellular transport, membrane assembly, secretion, and endocytosis.

This work has been presented in preliminary form (10).

MATERIALS AND METHODS

All chemicals, unless otherwise specified, were obtained from Sigma.

Retina Cell Culture. Dissociated chicken retina cells were obtained as described (11) and plated onto polylysine-treated, Formvar-coated gold grids in medium containing 10% fetal calf serum, 5% chicken embryo extract, and 85% Dulbecco's modified Eagle's medium at a density of 1×10^6 cells per 35-mm culture dish. After 3 days of culture, plating medium was replaced with serum-free supplemented medium (5 μg of insulin per ml, 100 μg of transferrin per ml, 20 nM progesterone, 100 μM putrescine, and 30 nM selenium in Dulbecco's modified Eagle's medium (12).

High-Voltage Electron Microscopy. Cells were fixed for 15 min at room temperature in 2.5% glutaraldehyde/0.1 M Hepes, pH 7.2. The cultures were rinsed in 0.1 M Hepes buffer, postfixed in 0.1% osmium tetroxide in distilled water for 10 min, rinsed with distilled water, stained with 1% uranyl acetate, dehydrated in ethanol (the 100% step contained molecular sieve), and critical point dried with carbon dioxide. The carbon dioxide was dried with a Tousimis no. 8782 filter on the siphon tank. The grids were then carbon coated and observed with the AEI-7 1 MeV microscope at the University of Wisconsin-Madison HVEM Facility.

RESULTS

Cultures of dissociated cells from embryonic chicken retina contained primarily well-attached, process-bearing neurons. After 3 days in culture, the cells tended to clump and to form small aggregates. This clumping, as well as non-neuronal cell proliferation, could be prevented by feeding the cells with a serum-free supplemented medium (12) 3 days after plating. Cultures that were switched to serum-free medium were morphologically more differentiated and had larger increases in protein and muscarinic cholinergic receptor levels after 6 days compared to those kept in serum-supplemented medium (not shown).

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Abbreviations: HVEM, high voltage electron microscopy; SER, smooth endoplasmic reticulum; CNS, central nervous system.

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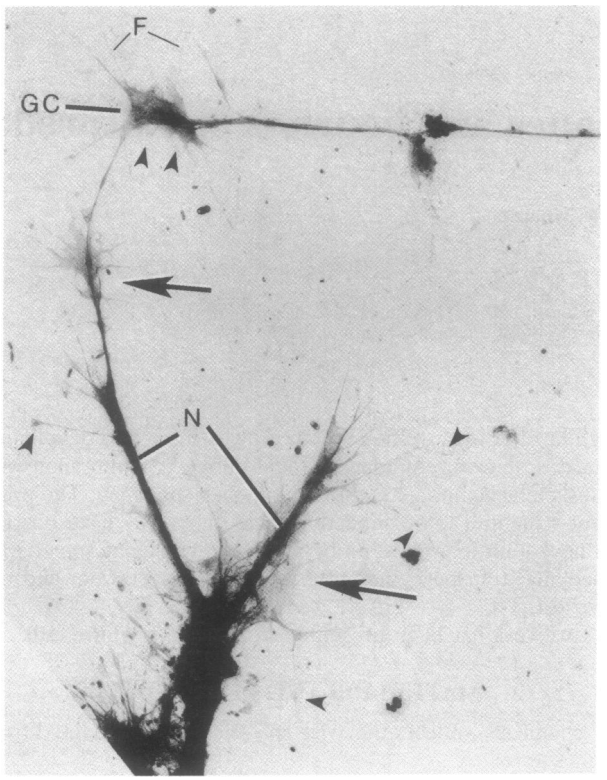

FIG. 1. Low-magnification HVEM image of embryonic retina neuron after 2 days in culture. Several neurites (N) extend from the cell body, and growth cones (GC) are apparent at the neurite tips. Regions with a flattened appearance (arrow) resembling that of growth cones also can be seen along the length of the neurites. Many filopodia (F) extend from the growth cone and flattened regions. Some of the filopodia have bead-like structures (arrowheads) at their tips or along their length. At the top left, there are two overlapping growth cones (double arrowheads) from two different cells. (×1,045.)

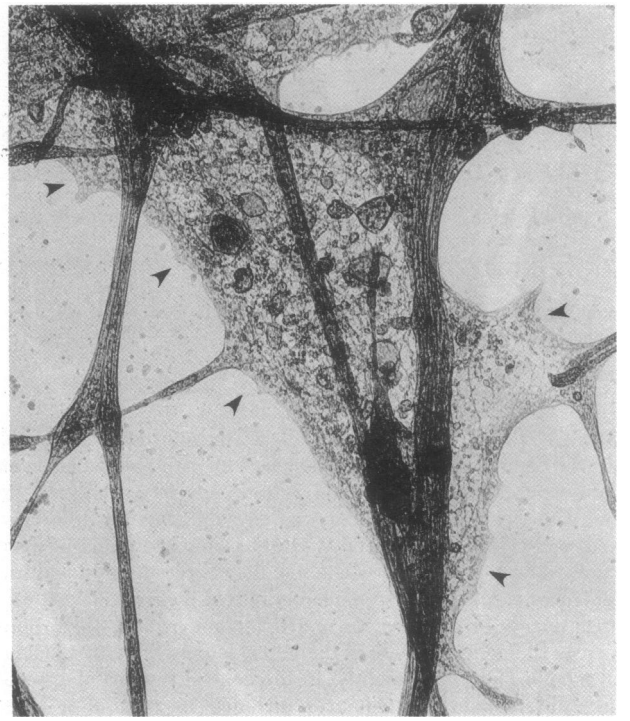

FIG. 2. HVEM image of 6-day-old retina culture. There is extensive arborization of neuronal processes which gives an intricate picture of overlapping neurites, growth cones, and filopodia. The growth cones contain large amounts of membranous organelles of various sizes as well as 20-nm structures, probably microtubules. Vesicles of more uniform size are prevalent at the edge of the growth cones (arrowheads). (×14,250.)

Fig. 1 shows a low magnification micrograph of a typical neuron after 2 days in culture. Neurites extend from the cell body and growth cones are apparent at the neurite tips. Regions with a flattened appearance resembling that of growth cones also can be seen along the length of the neurites. Many filopodia extend from the growth cone and flattened regions. In older cultures (Fig. 2), there was extensive arborization of neuronal processes, which gave an intricate picture of overlapping neurites, growth cones, and filopodia. Growth cones could be identified readily by using low-magnification images of the whole-mount specimens.

At higher magnification the most striking feature seen in the critical point dried growth cones was the prevalence of filamentous crosslinkers that interconnected the various organelles (Figs. 3–6). In the central regions of growth cones, the crosslinkers were of variable length (20–200 nm) and 5–9 nm in diameter. Each individual crosslinker was of uniform thickness along its length. Some crosslinkers appeared as side arms of microtubules, interconnecting the microtubules with large membranous structures, clusters of vesicles, and individual vesicles. The crosslinkers also interconnected the various membranous structures with each other. There appeared to be no systematic differences in width or appearance of the crosslinkers that interconnected different organelles.

At the periphery of growth cones, the crosslinkers were integrated within a filamentous network. The components of the network were again 5–9 nm in diameter, but their length was only 30–40 nm. The network linked together vesicles of approximately uniform size, 35–40 nm in diameter. Some parts of the filamentous network were parallel to the plasmalemma, and direct connections between the plasmalemma and the network could be seen.

Interconnections also were found between large membranous structures. In the center of the growth cone, there were many larger vesicles of irregular proportions and varying in size from 25 to 400 nm. These membranous organelles were connected to each other by 9- to 15-nm tapering structures. The sacs and connections may be part of the smooth endoplasmic reticulum (SER). In some cases, these irregular membranous structures were associated with nearby clusters of vesicles.

The vesicles connected by crosslinkers or associated with the presumptive structures were of various dimensions. At the central parts of growth cones, the vesicles showed a considerable range of diameters (25–75 nm) and often formed clusters. At the periphery, vesicles were not clustered and were more uniform in size (35–40 nm). In filopodia, the bulbous regions (diameters about 0.5 μm) found at the tips of filopodia usually were filled with these uniform-sized vesicles but the narrow connecting regions had few or none (Fig. 4). No vesicles were seen fusing with the membrane. The network of regular vesicles was seen only in neuronal growth cones and not in non-neuronal cells (Fig. 7). In young cultures (less than 3 days), there appeared to be fewer vesicles at the periphery of growth cones and more irregular vesicles in the central regions (not shown).

In thick sections (not shown), uniform-sized vesicles were observed at the edges of growth cones, although the dimensions of these vesicles and of other structures appeared slightly larger in the embedded preparations. The filamentous connections between vesicles, as well as the other filamentous crosslinkers, were detectable, but the clarity and resolution of

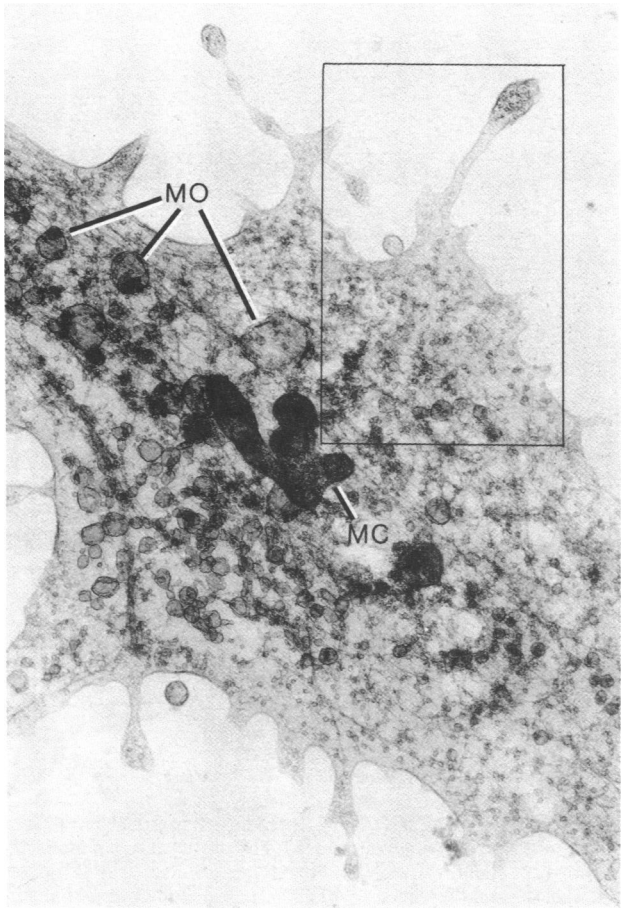

FIG. 3. Growth cone of a retina neuron at 4 days in culture. Closely packed microtubules (M) enter the proximal part of the growth cone at upper left and subsequently spread out. There are large amounts of membranous organelles (MO) resembling the sacs of the smooth endoplasmic reticulum (SER), clustered and nonclustered vesicles of various sizes, and mitochondria (MC). As in Fig. 2, at the periphery of the growth cone are many small vesicles interconnected by a filamentous network. Area in box is shown as stereo pair at higher magnification in Fig. 4. (×20,000.)

the images were drastically reduced compared to the whole-mount images.

DISCUSSION

The most striking feature of differentiating neurites examined by HVEM was the remarkable degree of interconnection seen in all parts of growth cones. We were able to detect this linkage because HVEM images of critical point dried, whole-mount specimens provide a three-dimensional perspective with great depth and clarity. Two major observations were particularly intriguing. (*i*) Throughout growth cones, there were abundant, nontapering filaments that crosslinked microtubules, SER, and vesicles of various sizes. (*ii*) At the periphery of growth cones and in filopodia, there was a distinct filamentous network that interconnected uniform-sized vesicles (35–40 nm) and that, in places, connected to the surface membrane.

In the central region of the growth cones, we found large numbers of irregularly shaped vesicles that were connected to each other by tapering 6- to 15-nm structures. These structures most likely were part of the SER. Previous serial section studies on neurites have shown the SER to vary in form from membrane-bound vesicles to thin filaments (13), although it has been suggested that the SER forms a continuous network of channels from the cell body to the nerve terminals (14, 15). We have not yet been able to determine whether the sacs seen in growth cones form a continuous channel of membrane-bound structures, although the narrow regions (6 nm) would seem too thin for the two lipid bilayers expected for a membranous channel. In addition to these irregular-shaped presumptive SER structures, we also saw many smaller, more spherical, and often clustered vesicles at the center of the growth cones. These vesicles were linked to each other and the SER and may represent structures that bud off from the SER (14). Some of them may represent the smooth vesicles that undergo retrograde transport (16, 17).

A second, and much more prevalent, type of linkage occurred between cytoskeletal components as well as membranous structures. This linkage was mediated by discrete, nontapering filaments 5–9 nm in diameter. We found no systematic differences in width or appearance of individual crosslinkers that connected different kinds of organelles, although the filamentous network at the edges of growth cones had shorter filaments (30–40 nm) than found in the central regions. Similar intricate lattices have been seen in whole-mount studies of other cells, although the crosslinkers observed, called "trabeculae" (9, 18, 19), were very irregular in appearance. It now appears that the irregularity of the trabeculae could arise as an artifact during critical point drying (20, 21). Further support for a network of discrete filaments rather than trabeculae was provided by a recent study of platinum replicas of freeze-dried fibroblast cytoskeletons (22).

The filamentous network not only links cytoskeletal components but also connects numerous 35- to 40-nm vesicles, especially at the periphery. Such interconnections seem likely to be involved in directing the intracellular transport of membrane material. Previous reports have mentioned the occasional presence in growth cones of unlinked vesicles of various sizes and types (6, 7, 23–25). Similarly, reports of microfilament meshwork in growth cones have not indicated linkages to vesicles (23, 24). However, the filamentous network in whole mounts observed by HVEM is more distinct than can be seen in thin sections. In addition, our preliminary data suggest that there were more vesicles present in the older cultures used in this study than in younger cultures and that the network was better established. It is possible that some of the growth cones we examined by HVEM were in the process of developing into more quiescent terminals. However, light microscopic observation of our 4- to 6-day-old cultures still revealed many motile growth cones.

Growth cones carry out a number of special functions, such as endocytosis (6, 7), directed movement of vesicles (8), and assembly of surface membranes (3–5); vesicle filament linkages may be important in these processes. It is especially interesting that the filamentous network adjoined the plasmalemma at places, suggesting a possible means for specific interaction of the vesicles with the surface membrane, either for addition or removal of membrane constituents. Membrane-associated actin-binding proteins, postulated to provide a linkage between microfilament-based structures and the surface membrane, have been found in neural tissue (26, 27), whereas actin and actin-binding proteins have been found in growth cones of cultured dorsal root ganglia neurons (28–30).

It is conceivable that the vesicles prevalent near the surface in more mature neurites represent synaptic vesicles. They were regular in size, and their dimensions approximated those of synaptic vesicles. The vesicles were more abundant in older cultures and they accumulated in the bulbous ends of filopodia. It should be emphasized that these bulbous ends appeared to be different from the mound-like areas observed by others (3,

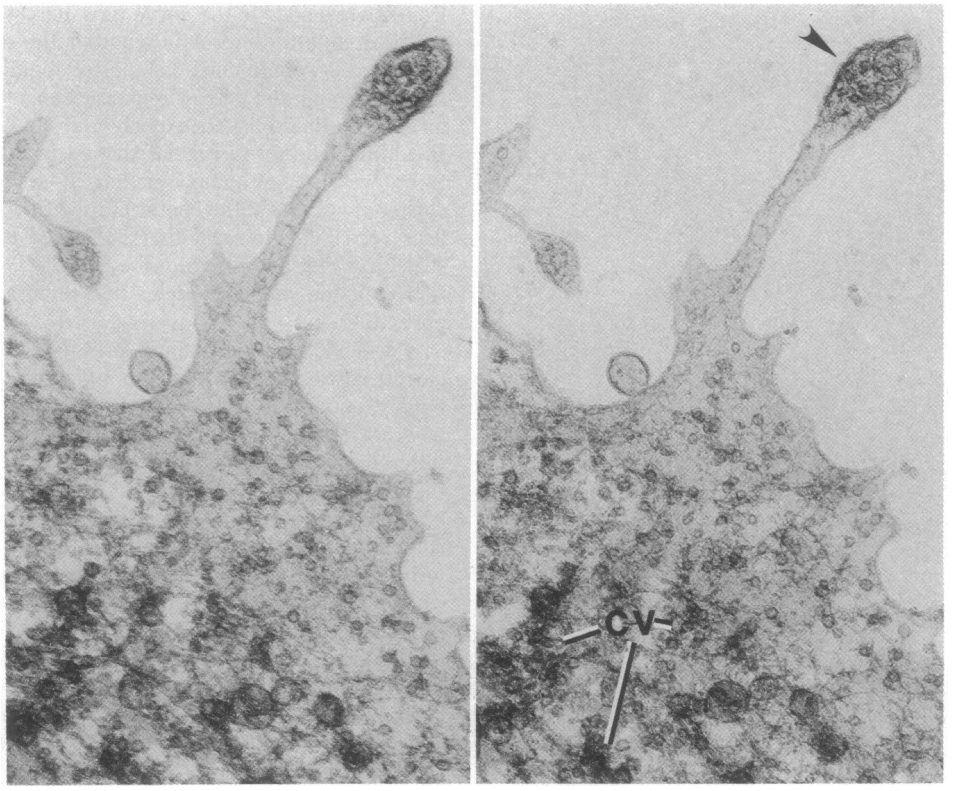

FIG. 4. Stereomicrograph of a filopodium at the periphery of the growth cone in Fig. 3 (region in box), shown at higher magnification. Toward the central part of the growth cone are clustered vesicles (CV), individual vesicles of larger size, and microtubules. These structures are connected to each other through slender crosslinkers. At the edge of the growth cone are small vesicles of uniform size (35–40 nm) interconnected by a filamentous network. The bulbous region of the filopodium (arrowhead) at the tip is filled with the small vesicles. (×40,000; the stereomicrographs should be viewed with a stereo print viewer which enlarges about 2 times.)

6). The mound-like areas typically contain nothing but the large irregular ovoid or tubular vesicles (75–150 nm in diameter). We rarely saw the mound-like structures in our preparations. The bulbous endings of the filopodia in our preparations contained 35- to 40-nm vesicles as well as fine filaments. The endings were about the size of small synaptic boutons and conceivably could be nascent presynaptic terminals. Growth cones of cultured sympathetic neurons contain adrenergic vesicles (25), and transmitter appears to be released from growth cones during early axonal contact of motor neurons with muscle cells (31–33).

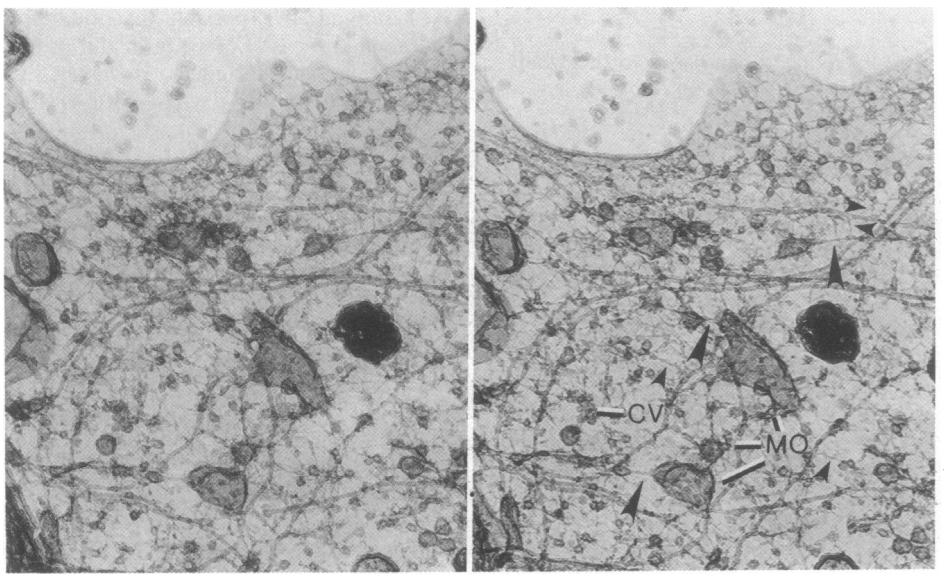

FIG. 5. Stereomicrograph of a growth cone from a neuron 6 days in culture, showing extensive crosslinkage among various organelles in the growth cone. Some crosslinkers appear as side arms (small arrowheads) of microtubules interconnecting the microtubules with large membranous structures, clustered of vesicles (CV), and individual vesicles. Large membranous organelles (MO) seem to be connected to each other by tapering structures (large arrowheads). (×40,000.)

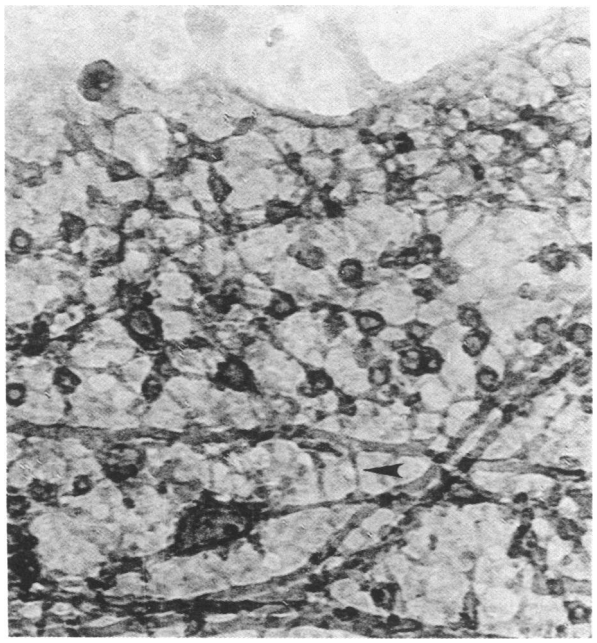

FIG. 6. High-magnification HVEM image of the periphery of the growth cone shown in Fig. 5. The crosslinkers are integrated within a filamentous network which links together vesicles of approximately uniform size. Some parts of the filamentous network are parallel to the plasmalemma, and direct connections between the plasmalemma and the network can be seen. At arrowhead, a crosslinker connects the microtubule with a possible SER sac. (×113,000.)

If the 35- to 40-nm vesicles we observed in growth cones were synaptic vesicles, their attachment to a filamentous network may provide structural order to mechanisms of vesicle assembly and movement in differentiating neurites. This orderly positioning of vesicles may persist in mature neurons, a possibility suggested by other recent work (34).

Whole-mount studies have been performed previously on growth cones of the dorsal root ganglia (29, 35), but our HVEM study provides clearer pictures of the high level of organization within growth cones and developing neurites. This result appears to be due to the use of proper critical point drying procedures (20) and the use of a low concentration (≈1%) of osmium tetroxide (21, 36). Our results with critical point dried cells together with other recent ultrastructural studies on axons (19, 37, 38) indicate that interconnections between cytoskeletal and membranous organelles are generally occurring. Other regions of neurons are likely to have similar levels of organization. Because functions such as specific membrane assembly, intracellular transport, secretion, and endocytosis are not unique to growth cones, the extensive linkages we have observed here may have implications for the development and function of cells in general.

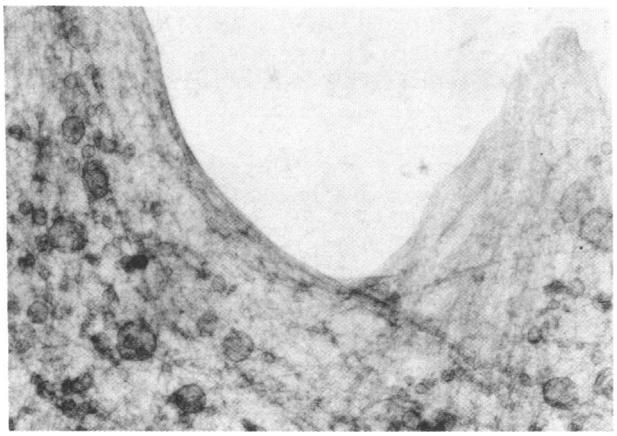

FIG. 7. HVEM image of non-neuronal cell in retina culture. Fine filaments form a dense network as well as bundles at various places. In contrast to the growth cone in Fig. 3, note the absence of uniform-sized 35–40 nm vesicles in these non-neuronal cells. (×19,000.)

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